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Filaricidal Activity of CGP 20376 against *Brugia malayi* Microfilariae, Larvae, and Adults

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ABSTRACT: The macrofilaricidal drug CGP 20376 was evaluated for its capacity to kill all stages of subperiodic *Brugia malayi* at various doses in inbred *Meriones unguiculatus* (jirds). Killing of microfilariae (MF) and infective stage larvae (third-stage larvae [L3's]) was also studied at various drug concentrations in vitro. Studies in vitro were performed in 24-well culture plates to evaluate drug concentrations ranging from 1,000 to 0.01 μg/ml. Culture wells containing 500 MF each or 20 L3's each were dosed with 10-fold dilutions of CGP 20376 suspended in dimethyl sulfoxide (DMSO) and serum-free medium. Three replicates of each experiment were performed. MF were killed within 2 hr at drug concentrations of 1,000 and 100 μg/ml. Killing reached 100% by 24 hr with 0.1 μg/ml of the drug, whereas at the lowest concentration, 0.01 μg/ml, complete killing required 35 hr. MF in medium only or in medium with DMSO remained viable after 35 hr in culture. For L3's, drug concentrations of 1000 and 100 μg/ml killed 100% of the larvae by 2.5 hr and by 15 hr with 10 μg/ml. In 1 μg/ml, 50% were dead by 20 hr and 90% by 25 hr. However, at this concentration, a few L3's remained alive and sluggishly motile for 165 hr. The effects of CGP 20376 on MF, adults, and developing larvae were evaluated in groups of age-matched inbred male jirds. A single dose of 25 mg/kg of CGP 20376 was more than 99% effective against fourth-stage larvae in vivo. Higher doses were required to kill adult worms within lymphatics.

KEY WORDS: CGP 20376, filaricidal drug, *Brugia malayi*, jirds, filariasis.

Lymphatic filariasis caused by *Wuchereria bancrofti* and *Brugia* species remains a serious health problem affecting more than 78 million people in tropical and subtropical regions of the world (World Health Organization, 1992). The lack of safe and reliable chemotherapeutic agents against larvae and adult worms has been a major hindrance to successful filarial control efforts. Diethylcarbamazine (DEC) has been the drug of choice for treatment of *Wuchereria* and *Brugia* infections for several decades; however, DEC is primarily a microfilaricide that is administered in large doses over several days, and its use is often plagued by serious side effects. The efficacy of single doses of DEC has recently been evaluated (Kimera et al., 1985; Paniker et al., 1991; Cartel et al., 1992; Kazura et al., 1993; Mataika et al., 1993; Shenoy et al., 1993; Dreyer et al., 1994). Ivermectin, a newer antifilarial drug that kills microfilariae and suppresses microfilaremias is currently undergoing field trials (reviewed in Ottesen and Campbell, 1994; Chodakewitz, 1995).

Several compounds that are chemically classed as isothiocyanates and their derivatives have antifilarial activity (Subrahmanyam, 1987; Townson et al., 1990). One such compound, CGP 20376, has been evaluated in vitro against *Onchocerca volvulus* (Strote, 1989) and *Litomoside carinii* (Davies et al., 1989) and in several animal models, including *O. volvulus*–infected rats (Strote, 1989), *Dipetalonema*-infected chimpanzees (Moysan et al., 1988), and *B. malayi* in monkeys (Mak et al., 1990), rats (Zahner et al., 1990), and jirds (Chandrashekar et al., 1990, 1991). Here we report on the efficacy of CGP 20376 against *B. malayi* microfilariae (MF) and infective stage larvae (third-stage larvae [L3's]) in vitro as well as larval and adult stages in jirds.

Materials and Methods

Subperiodic *B. malayi* was used throughout these studies. Infected jirds as well as stock *Aedes aegypti* eggs for maintenance of the entire life cycle were obtained from the U.S.–Japan Cooperative Filariasis Program repository (University of Georgia, Athens, Georgia). Inbred *Meriones unguiculatus* were obtained from Tumblebrook Farms (West Brookfield, Massachusetts). L3's were harvested from *Ae. aegypti* 14 days after the infecting blood meal as previously described (Yates et al., 1994), except that mosquitos were surface-sterilized in 95% ethanol before L3's were collected. MF were collected by syringe and needle from jirds with intraperitoneal infections (McCall et al., 1973).

In vitro studies were performed in triplicate in 24-well culture plates to evaluate the effects of drug concentrations ranging from 1,000 to 0.01 μg/ml on L3's and MF. Cultures were incubated at 37°C with 95% relative humidity and 5% CO_2 in air. For the experi-

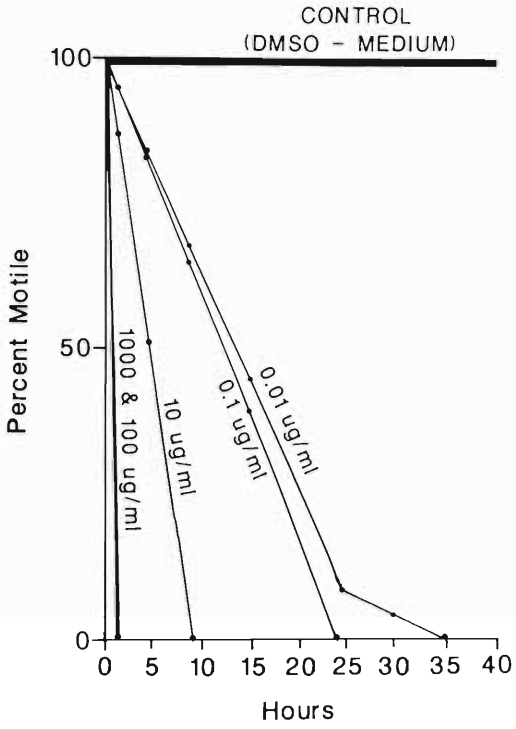

Figure 1. In vitro effect of CGP 20376 on *Brugia malayi* MF.

ments with MF, culture wells each containing medium F12 and 500 MF were dosed with 10-fold dilutions of CGP 20376 suspended in dimethyl sulfoxide (DMSO) and medium. Control culture wells contained medium with the maximum DMSO concentration used or medium only. All wells contained a final volume of 1 ml and a pH of 7.2. For the in vitro experiments with L3's, culture conditions were similar except that 20 larvae were placed in each well and doses of 0.5 and 0.05 μg/ml were also evaluated. Mortality was determined on the basis of absent motility and the apparent loss of structural integrity. Larvae considered dead by these criteria did not regain motility after washing and reincubation in fresh medium.

The in vivo effects of CGP 20376 on developing larvae, adult worms, and MF in *M. unguiculatus* were evaluated in groups of age-matched inbred male jirds. Three types of experiments were designed to evaluate the following: (a) the effect of a single dose (25 mg/kg, by stomach tube) on fourth-stage larvae (L4's) and the level of residual killing 3 wk after such a single dose; (b) the effect on development of microfilaremia after 1 or 2 doses (25 mg/kg) given at various intervals after infection; and (c) the effect of 2-×-25-mg/kg doses given to jirds with stable, patent infections. The jirds were 8 wk old at the beginning of each study. To facilitate drug delivery by stomach tube, CGP 20376 was suspended in DMSO and diluted in RPMI-1640. Jirds receiving 2 doses were given 6 hr rest between doses. In each experiment, the drug was suspended immediately prior to use. Sham-treated control jirds received doses of the vehicle (RPMI-1640 with 1.8% DMSO) alone. Blood for MF counts and serological testing was collected from the retroorbital venous plexus. Serum from selected jirds was assayed for anti-*Brugia* immunoglobulins by enzyme-linked immunosorbent assay. Jirds were necropsied and adult worms were enumerated as described previously (Yates and Higashi, 1985).

Results

Brugia malayi MF and L3's were highly sensitive to the filaricidal activity of CGP 20376 in vitro without complement or added serum. MF were killed within 2 hr at drug concentrations of 1,000 and 100 μg/ml (Fig. 1). Killing reached 100% by 24 hr with 0.1 μg/ml of the drug, whereas complete killing required 35 hr at the lowest concentration, 0.01 μg/ml. MF in medium only or in medium with DMSO were more than 99% viable after 35 hr in culture. For L3's, drug concentrations of 1,000 and 100 μg/ml killed 100% of the larvae by 2.5 hr in culture and by 15 hr all were killed with 10 μg/ml (Fig. 2). In 1 μg/ml, 50% were dead by 20 hr increasing to 90% by 25 hr. Interestingly, 6 L3's in 3 different culture wells remained alive and sluggishly motile for 165 hr in the 1-μg/ml concentration. Drug concentrations of 0.5 and 0.05 μg/ml had no apparent effect. Larvae in medium only and medium containing DMSO remained active and normal in appearance up to the end of the 170-hr culture period. The maximum serum concentration in healthy human volunteers given single doses of 8 mg/kg of CGP 20376 during toxicity testing by Ciba-Geigy was in the range of 1 μg/ml (Dr. H. P. Streibel, Ciba-Geigy Limited, Basel, Switzerland, pers. comm.).

The effects of CGP 20376 on developing L4's and potential residual activity of the drug 3 wk after treatment were evaluated in 3 groups of age-matched inbred male jirds (14 animals per group). At the outset, 1 group of jirds was given 25 mg/kg of CGP 20376 each by stomach tube. Three weeks later, all 3 groups were infected with *B. malayi* by subcutaneous injection of 75 infective stage larvae per jird. After an additional 3 wk, 1 of the previously untreated groups of jirds was treated with 25 mg/kg each of the compound. The jirds were then kept for another 15 wk before infections were evaluated in terms of microfilaremias and recovery of adult worms from the lymphatics and viscera. CGP 20376 was very effective against L4's of *B. malayi,* treated after infection (Table 1). None of the 14 jirds in this group were microfilaremic after 18 wk of poten-

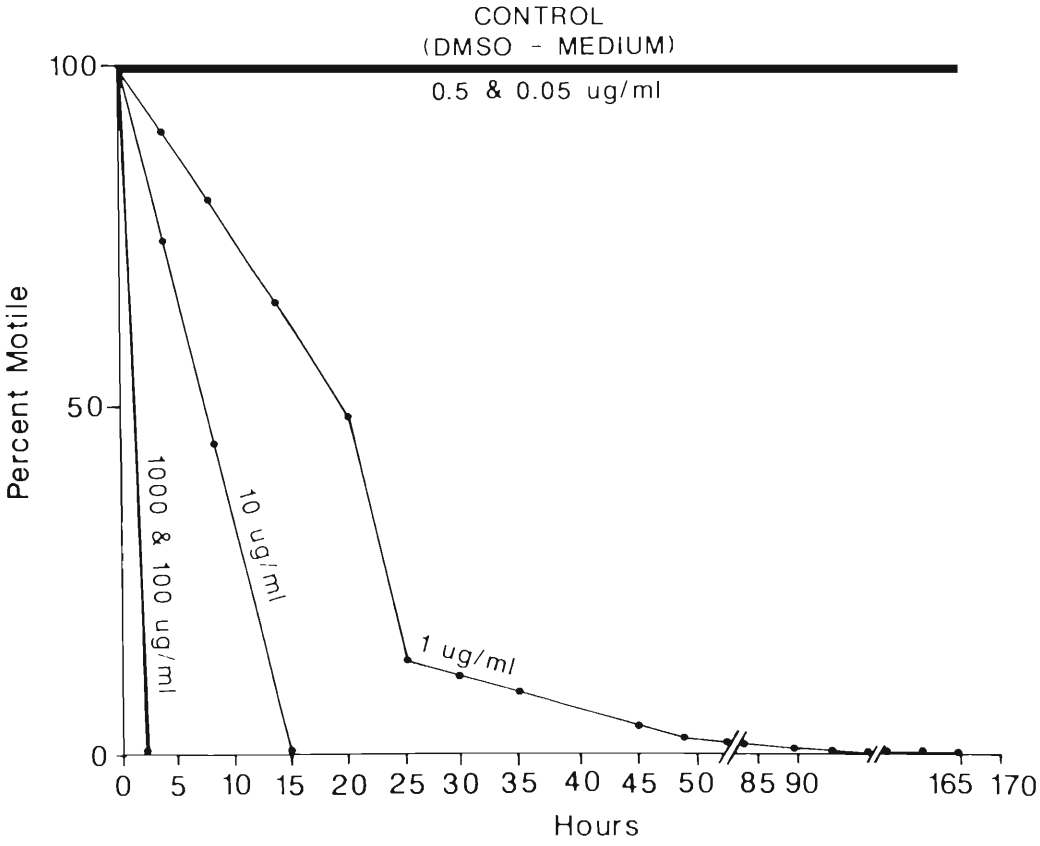

Figure 2. In vitro effect of CGP 20376 on *Brugia malayi* L3's.

tial development and at necropsy only 3 worms were found in the lymphatics or viscera of these jirds. In contrast, the other groups of jirds were heavily and similarly infected (Table 1). Normal development of *B. malayi* in jirds that were infected 3 wk after treatment with the drug was apparent. Microfilaremias were detected in 10 of these animals, and adult worms were found in the lymphatics of every jird in this group. Indeed, these findings were not significantly different from the sham-treated control group. The results of this experiment indicated that a single dose of 25 mg/kg of CGP 20376 was highly effective against the L4 stage, although a few worms survived this treatment. Filaricidal levels of the compound did not persist in the jirds 3 wk post-treatment.

From our preliminary experiments, it was apparent that a single dose protocol using 25 mg/kg as suggested by the drug manufacturer provided less than total clearance of worms from some jirds but seemed to produce amicrofilaremic infections in those jirds that harbored residual worms. In our early studies, treatments had always been given when the parasites were at the L3 or L4 stage and substantial but incomplete killing was noted (e.g., Table 1). Therefore, it was of interest to evaluate the effect on development of microfilaremia after 1 or 2 doses (25 mg/kg) given at different times in the course of infection. To that end, 144 jirds (12 groups of

Table 1. Effect of CGP 20376 (1 dose, 25 mg/kg) given to jirds 3 wk before or 3 wk after *Brugia malayi* infection.

Treatment	No. of jirds	No. of jirds with microfilaremia*	Mean No. of worms recovered
3 Wk before	14	10/14	7.4 (SD 4.11)
3 Wk after	14	0/14	0.2
Sham treatment	14	8/14	10.1 (SD 5.72)

* MF counts and necropsy 18 wk after infection.

Table 2. Effect on microfilaremia of CGP 20376 treatment (group A, 1 dose, 25 mg/kg; group B, 2 doses, 25 mg/kg) given to jirds at various intervals after *Brugia malayi* infection.

Treatment	No. of jirds		No. of jirds with microfilaremia			
			At 5 mo		At 10 mo	
	A	B	A	B	A	B
Sham treatment	12	12	12/12	12/12	11/12	12/12
After 7 days	12	12	1/12	0/12	1/12	0/12
After 20 days	12	12	3/12	0/12	3/12	0/12
After 32 days	12	12	2/12	0/12	3/12	0/12
After 42 days	12	12	3/12	0/12	5/12	0/12
After 100 days	12	12	5/12	0/12	9/12	0/12

12 jirds each) were infected with 100 *B. malayi* L3's each by the subcutaneous route. Ten groups were treated at 5 different times postinfection (PI); half the groups with the single-dose protocol (25 mg/kg) and half with 2 doses given 6 hr apart for a total of 50 mg/kg. As a control, 2 groups were sham-treated, one with a single dose of the vehicle only and the other with 2 doses of the vehicle. The sham treatments were given 6 days after infection. Because of the large number of L3's required for this study, it was necessary to infect the groups of jirds on 2 occasions. It was convenient to divide the experiment in half so that all jirds receiving the same treatment dose were infected on the same day. The 5 treatment times were chosen to correlate with various stages in the course of worm development. Treatments at 7 days of development were directed against the L3 stage a few days before the molt to L4, which occurs at about day 9 or 10. Treatments at days 20 and 32 PI were directed at the mid- and late L4, respectively, with the molt to immature adults (L5's) occurring between days 35 and 40. L5's were the target of treatments on day 42 PI, whereas day 100 treatments were directed at mature adults. Microfilaremias were evaluated at 5 and 10 mo PI. Two doses of 25 mg/kg given 6 hr apart prevented microfilaremia regardless of the developmental stage of the worms at the time of treatment (Table 2). However, with the single-dose protocol, there were patent infections in every treated group. All of the sham-treated jirds developed patent infections.

With an adequate treatment dose for preventing microfilaremia established at 50 mg/kg given in 2 equal doses, a third study was conducted to determine the effectiveness of this treatment in jirds with stable, patent infections. Sixteen jirds were infected with 100 L3's each. These jirds developed microfilaremias by 4 mo PI and maintained moderate MF levels (13–366 MF/20 μl of venous blood) until 10 mo PI. At that time, the jirds were randomly divided into 2 equal groups and either treated or given a sham treatment. MF were cleared from the blood of the treated jirds by 1 wk posttreatment and did not return for 6 mo (Table 3), at which time the jirds were necropsied. Microfilaremias persisted in the sham-treated group over the same time period. At necropsy, the CGP 20376–treated jirds were free of detectable worms whereas the sham-treated jirds were heavily infected with adult worms (Table 3). Correspondingly, serum antibody levels decreased slightly after treatment but persisted at high levels 6 mo after treatment.

Table 3. Effect of CGP 20 376 (2 doses, 25 mg/kg, each) given to jirds with stable, patent *Brugia malayi* infections.

Treatment	No. of jirds	Microfilaremia posttreatment			Mean adults recovered
		1 wk	3 mo	6 mo	
Sham treatment after 10 mo	8	8/8	8/8 (SD 16.3)	7/8	21.2
After 10 mo	8	0/8	0/8	0/8	0

Discussion

We have shown that CGP 20376 kills *B. malayi* MF and L3's in vitro without the aid of complement, cells, or other host factors at various drug concentrations. Furthermore, killing occurred at drug concentrations that were similar to serum levels seen in healthy human volunteers who had taken a single dose of the compound during toxicity testing conducted by the drug's manufacturer (Dr. H. P. Streibel, Ciba-Geigy Limited, Basel, Switzerland, pers. comm.). These

results are consistent with in vitro studies of CGP 20376 against *O. volvulus* L3's and L4's, *L. carinii* MF and adults, and *B. malayi* MF (Davies et al., 1989; Strote, 1989; Zahner et al., 1991). We have also confirmed the utility of DMSO as a carrier in place of ethanol (Davies et al., 1989).

Studies in jirds clearly showed the efficacy of this drug against MF, all larval stages, and adult worms established in the lymphatics. Treatment with 25 or 50 mg/kg was well tolerated by jirds and no untoward side effects were noted. However, hepatotoxicity has been observed in humans over the course of drug trials with this compound (Mak et al., 1991; Kohler et al., 1992). A single dose of 25 mg/kg of body weight was suggested to us as a starting point for treatment of *B. malayi* in jirds by the compound's manufacturer. This treatment was effective against the L4's but did not provide a complete clearance of worms. Treatment with a total dose of 50 mg/kg of body weight given in 2 oral doses 6 hr apart, however, provided apparently total killing of worms regardless of the developmental stage of the parasites. Previous studies showed similar results in *B. malayi*–infected jirds, however, were not as extensive and typically utilized carboxymethyl cellulose to solubilize the drug (Chandrashekar et al., 1991). Multiple doses of the drug have also been effective against *B. malayi* in *Mastomys natalensis* (Zahner et al., 1988). The killing of adult worms in jirds with stable, patent infections was substantiated by the loss of microfilaremia and its continued absence for 6 mo, as well as the absence of worms at necropsy.

Other filaricidal compounds have been shown to produce a residual prophylactic effect after treatment that may persist for months (Chusattayanond and Denham, 1984), and in some cases experimental treatments with antifilarial drugs apparently may lead to enhanced immunoresponsiveness and resistance to future infections (McCall et al., 1978; Blair and Cambell, 1981; Grieve et al., 1988). From our studies, it is clear that CGP 20376 produced insignificant residual chemoprophylaxis against *B. malayi* in jirds 3 wk posttreatment. Enhanced posttreatment immunoresponsiveness was not evaluated.

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